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Eukaryotic initiation factor 4AI interacts with NS4A of Dengue virus and plays an antiviral role

Xiaoyan Chen [a, b], Jun Xia [a, b], Qirui Zhao [a, b], Yi Wang [a, b], Jingyu Liu [c], Lianqiang Feng [a, b], Junfang He [a, b, *], Ping Zhang [a, b, *]

[a] Department of Immunology, Institute of Human Virology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou 510080, China
[b] Key Laboratory of Tropical Diseases Control (Sun Yat-sen University), Ministry of Education, Guangzhou 510623, China
[c] Guangdong Inspection and Quarantine Technology Center, Guangzhou 510080, China

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ABSTRACT

Dengue virus (DENV) is a mosquito-borne *flavivirus* that causes the most prevalent diseases in tropical and subtropical regions. DENV utilizes host factors to facilitate its replication, while host cells intend to restrain virus replication. NS4A of DENV has been implicated to play a crucial role during viral replication. To identify more cellular proteins that are recruited by NS4A, we carried out a tandem affinity purification assay. The mass spectrometry data revealed that human eukaryotic initiation factor 4AI (eIF4AI) was one of potential NS4A-interacting partners. Co-immunoprecipitation data confirmed the interaction between NS4A and eIF4AI, and both the N-terminal ATP-binding domain and C-terminal helicase domain of eIF4AI were involved in their association. Functionally, silencing of eIF4AI by RNAi significantly increased the replication level of DENV1, DENV2 and DENV3. And knockdown of eIF4AI markedly attenuated the phosphorylation of protein kinase regulated by dsRNA-activated (PKR) and eIF2α induced by DENV2 infection. Collectively, these data suggested that a potential role of NS4A in antagonizing host antiviral defense is by recruiting eIF4AI and escaping the translation inhibition mediated by PKR.

1. Introduction

Dengue virus (DENV), a member of the *genus Flavivirus*, includes four serotypes (DENV1, DENV2, DENV3 and DENV4) [1]. Infection of DENVs may cause dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS) [2]. Due to the warming climate, the range and rate of DENV infection are increasing [3]. The genome of DENVs is a positive single-strand RNA and can be translated into a polyprotein that is subsequently cleaved by cellular and viral proteases into three structural proteins (C, capsid; prM, membrane; E, envelope) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). The NSs are the major proteins to execute subsequent events of viral replication, including RNA synthesis, protein translation and viral packaging.

Among these NS proteins, NS4A, a small transmembrane protein, plays an indispensable role in viral replication [4]. NS4A is a component of replication complex (RC), with its C-terminus colocalized with other NS proteins and viral dsRNA in the ER lumen [5,6]. To elucidate the detailed molecular mechanism of NS4A role, several groups including us have recently attempted to identify cellular proteins that interact with NS4A [7–10]. These work demonstrated that by interacting with NS4A, vimentin plays a role in the viral RC construction [9] and polypyrimidine tract-binding protein (PTB) regulates the viral negative strand RNA synthesis [7,8].

Our previous work purified several NS4A-interacting proteins through the tandem affinity purification (TAP) approach [10], and current study further investigated these proteins by Mass spectral analysis. We identified a potential candidate, eukaryotic initiation factor 4AI (eIF4AI). EIF4AI is a member of the DEAD box RNA helicase family. EIF4AI structurally consists of an N-terminal ATP-binding domain and a C-terminal helicase domain, and functions as a eukaryotic initiation factor. So far, eIF4AI has been indicated to facilitate the replication of some viruses, including influenza virus,

* Corresponding authors. Key Laboratory of Tropical Diseases Control (Sun Yat-sen University), Ministry of Education, Guangzhou 510623, China.
E-mail addresses: hejf@mail.sysu.edu.cn (J. He), zhangp36@mail.sysu.edu.cn (P. Zhang).

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Junin virus, Seneca valley virus, encephalomyocarditis virus (EMCV), and foot-and-mouth disease virus (FMDV) [11–16], while translation initiation of adenovirus, Sendai virus, and Cauliflower mosaic virus do not require the assistance of eIF4AI [17]. Nonetheless, the role of eIF4AI in the replication of dengue virus has not been well elucidated.

In this study, we have investigated role of eIF4AI in DENV replication. Unexpectedly, we found that eIF4AI played an antiviral role in the replication of several Dengue virus serotypes. Moreover, eIF4AI was involved in the activation of protein kinase regulated by dsRNA (PKR) and eIF2α. These data suggested that the association of NS4A with eIF4AI might present a strategy for virus to escape the host antiviral defense.

2. Materials and methods

2.1. Cell culture

Mosquito cell line C6/36 (ATCC; CRL-1660) and human lung carcinoma epithelial cells (A549) were cultured in RPMI-1640 (Gibco, CA) with 10% (v/v) fetal bovine serum (FBS) (Gibco, CA) at 28 °C or in DMEM with 5% FBS at 37 °C respectively. The media were added with 1% sodium pyruvate, 100 units/ml of streptomycin and penicillin (Invitrogen, CA).

2.2. Plasmids

In the mixture of PCR to amplify full-length or truncated fragments of eIF4AI gene, cDNAs prepared from A549 cells were used as template. The reverse primers included a FLAG-encoding sequence (primer sequences were listed in Table 1). The amplified fragments were purified, digested by indicated restriction enzymes and cloned into pSG5 vector.

2.3. Viruses and virus titration

The DENV1 Hawaii strain, DENV2 New Guinea C (NGC) strain and DENV3 H87 strain were presented by Guangzhou Centers for Disease Control. The virus stocks were prepared as described previously [18]. Briefly, DENVs were inoculated in C6/36 cells at a multiplicity of infection (MOI) 1. The supernatants were collected when cytopathic effect appeared and the cellular debris was removed by centrifugation. DENV stocks were titered on C6/36 cells and stored at −80 °C as described previously [19].

2.4. RNAi

The sequences of two siRNAs targeting eIF4AI were GGACCAGAUCUAUGACAUAUU and GCCCAAUCUGGGACUGGGA (Invitrogen, CA). A control siRNA with scrambled sequence was used as negative control (siNC). A549 cells were seeded into 12-well plate (8×10^4 per well). siRNAs were transfected into cells at a concentration of 2 μM by Lipofectamine 2000 (Invitrogen, CA) as described previously [19]. At 48 h post transfection, cells were harvested or used for further assay.

2.5. Real-time PCR

Total RNAs were prepared from cells using TRIzol reagent (Invitrogen, CA), and used for reverse-transcription. cDNAs were used for quantative real-time PCR to measure the viral RNA expression levels. The sequences of primers specific for DENV1, DENV2 and DENV3 were listed in Table 1. The relative mRNA levels were normalized to GAPDH.

2.6. Western blot

Whole cell extracts were prepared as described previously [19]. Briefly, cells were washed by PBS and lysed in RIPA buffer. Proteins were separated on 10% SDS-PAGE and transferred onto nitrocellulose membranes. Then membranes were blocked in PBS with 5% silk-milk and incubated with indicated primary antibodies, followed by incubation of secondary antibodies. Primary antibodies included anti-PKR, anti-E protein, anti-eIF2α (Santa Cruz Biotechnology, CA), anti-phospho-PKR (Epitomics, CA), anti-eIF4AI (Thermo Fisher, IL), anti-phospho-eIF2α (Cell signaling Tech, CA), anti-NS5, anti-HA anti-β-actin (Sigma, MO), and anti-FLAG (Beverly). Secondary antibodies were IRDye 800 CW conjugated anti-rabbit IgG or IRDye 680 CW conjugated anti-mouse IgG (LI-COR, NE). Then, the membranes were scanned by Odyssey infrared imaging system and analyzed by Quantity One software.

2.7. Co-immunoprecipitation assay

A549 cells (6×10^5 per well, 6-well plate) were co-transfected with plasmids expressing NS4A-HA, together with eIF4AI-FLAG, eIF4AIN-FLAG or eIF4AIC-FLAG fusion proteins by Lipofectamine 2000 (Invitrogen, CA). At 24 h post transfection, whole cell extracts

Table 1
Sequences of primers used in PCR.

Gene name	Sequence (5′-3′)	Restriction site
eIF4AI-FLAG	5F:CGGAATTCATGTCTGCGAGCCAGGATTCCCGAT	EcoRI
	3R:CCCAAGCTTCTACTTATCGTCGTCATCCTTGTAA	HindIII
	TCACCACCACCTCAGATGAGGTCAGCAACATTGA	
eIF4AIN-FLAG	5F:CGGAATTCATGTCTGCGAGCCAGGATTCCCGAT	EcoRI
	3R:CCCAAGCTTCTACTTATCGTCGTCATCCTTGTAAT	HindIII
	CACCACCACCAAGAATCCGAATGGGGTCCCTCA	
eIF4AIC-FLAG	5F:CGCGGATCCATGCTTGTCAAGAAGGAAGAGTTGAC	BamHI
	3R: CCCAAGCTTCTACTTATCGTCGTCATCCTTGTAAT	HindIII
	CACCACCACCTCAGATGAGGTCAGCAACATTGA	
GAPDH	5F:CCTTCCGTGTCCCCACTG	
	3R:CGCCTGCTTCACCACCTTC	
DENV1	5F:AAGCTCCCACGTCGGAAATA	
	3R:TGTCTGTTCCAAGTCTCTTGAG	
DENV2	5F:CTACAGAGTGGCAGCCGAAGGCATCAACTA	
	3R:ATTCCTTTAGCGCCAGTGGGTCAGAGTAGA	
DENV3	5F:AGGCATCAACCGCTGAAGC	
	3R:TTCCTGTTCCAAGTTGGTGTTT	
eIF4AI	5F:GCGTCATCGAGAGTAACTGGAATG	
	3R:GATACAAGGTAGAATGGCTCGCTG	

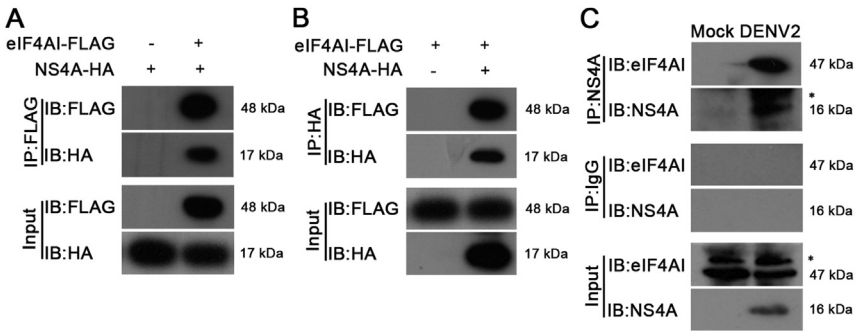

Fig. 1. NS4A interacted with eIF4AI. A549 cells were co-transfected with plasmids expressing pSG5-NS4A-HA or pSG5-eIF4AI-FLAG. Cell extracts were prepared at 24 h post-transfection and used for co-IP using FLAG (A) or HA antibody (B). Protein complexes were separated on SDS-PAGE and detected by Western blotting using HA and FLAG antibody. pSG5 vector was used as a negative control. (C), A549 cells were mock or infected with DENV2 at an MOI 2. Cell extracts were prepared at 24 h post-infection and used for co-IP using NS4A antibody. *: nonspecific band.

were prepared for co-immunoprecipitation assay using anti-HA or anti-FLAG agarose (Sigma, MO) as described previously [19].

To perform co-immunoprecipitation assay detecting the interaction between endogenous eIF4AI and viral NS4A, A549 cells were mock or infected with DENV2 at an MOI 2. At 24 h p.i., whole cell extracts were collected in RIPA lysis buffer and used for co-immunoprecipitation assay using Protein A/G agarose (Millipore, MA) as described previously [19]. Precipitated proteins were analyzed by Western blot.

2.8. Statistical analysis

All statistical analysis of viral RNA levels or viral titers were performed with an unpaired, two-tailed Student's *t* test. Data were presented as mean ± SEM from at least three independent experiments.

3. Results

3.1. EIF4AI was associated with DENV2 NS4A protein

Our previous study to isolate the potential NS4A-interacting proteins by TAP assay has identified two protein bands with molecular mass of 50 kDa and 30 kDa [10]. The 50 kDa protein band was sequenced by LC-MS/MS system revealing several proteins with high hit scores. Among them, we focused on eIF4AI in this report. To validate the interaction between NS4A and eIF4AI, we carried out the co-immunoprecipitation (co-IP) assay. A549 cells were co-transfected with plasmids expressing eIF4AI-FLAG and NS4A-HA fusion proteins, and harvested for co-IP assay using antibodies against HA or FLAG respectively. The FLAG antibody co-precipitated with both of eIF4AI and NS4A protein (Fig. 1A). Similarly, the HA antibody was able to co-immunoprecipitate with NS4A or eIF4AI (Fig. 1B). To further validate the interaction of viral NS4A with endogenous eIF4AI, we conducted the co-IP assay using whole cell lysates from mock- or DENV2-infected cells. Western blot data showed that the NS4A antibody co-immunoprecipitated with both of eIF4AI and NS4A protein (Fig. 1C). In contrast, NS4A or eIF4AI was not detected in immunoblots of control using the rabbit IgG. These data indicated that NS4A was associated with cellular eIF4AI during viral infection.

3.2. Both the N-terminal and C-terminal domains of eIF4AI were associated with NS4A protein

To map which domain of eIF4AI is involved in its interaction with NS4A, we generated two constructs expressing eIF4AI truncates lacking the C-terminal helicase domain (pSG-eIF4AIN, 28 kDa) or the N-terminal ATP binding domain (pSG-eIF4AIC, 20 kDa) respectively (Fig. 2A). A549 cells were co-transfected with plasmids expressing NS4A-HA and eIF4AIN or eIF4AIC, and cell lysates were harvested for co-IP assay. Surprisingly, the NS4A protein was coprecipitated with both of full length eIF4AI (FL), eIF4AIN (N) and eIF4AIC (C) (Fig. 2B), suggesting that both of eIF4AI

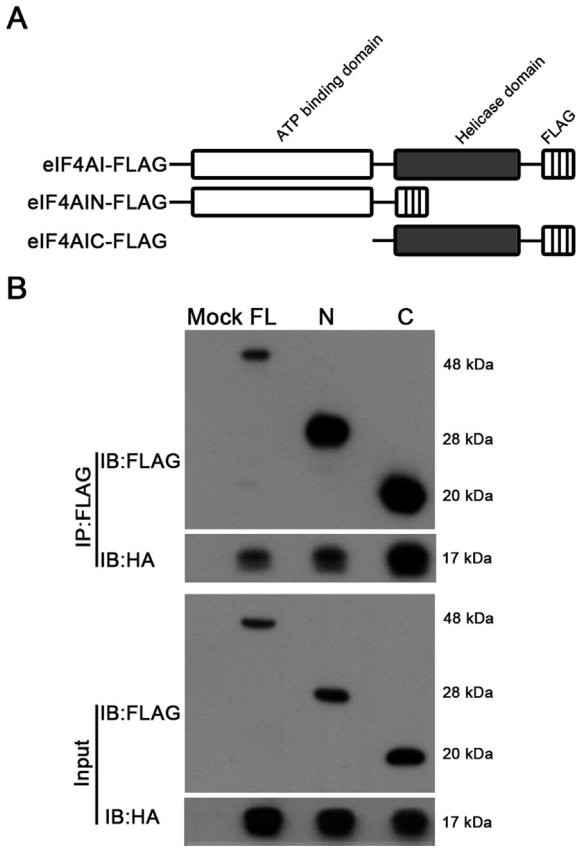

Fig. 2. Both the N-terminal ATP binding domain and C-terminal helicase domain of eIF4AI interacted with NS4A. (A), Schematic presentation of full length eIF4AI and truncates that expressed N-terminal ATP-binding domain or C-terminal helicase domain. (B), A549 cells were cotransfected with the constructs expressing NS4A-HA and full length (FL) or truncated forms of eIF4AI (N: N-terminal domain, C: C-terminal domain). Cells were harvested at 24 h post transfection for co-IP assay using FLAG antibody. The immunoprecipitated pellets were subjected to Western blot. Data were representative of three independent experiments.

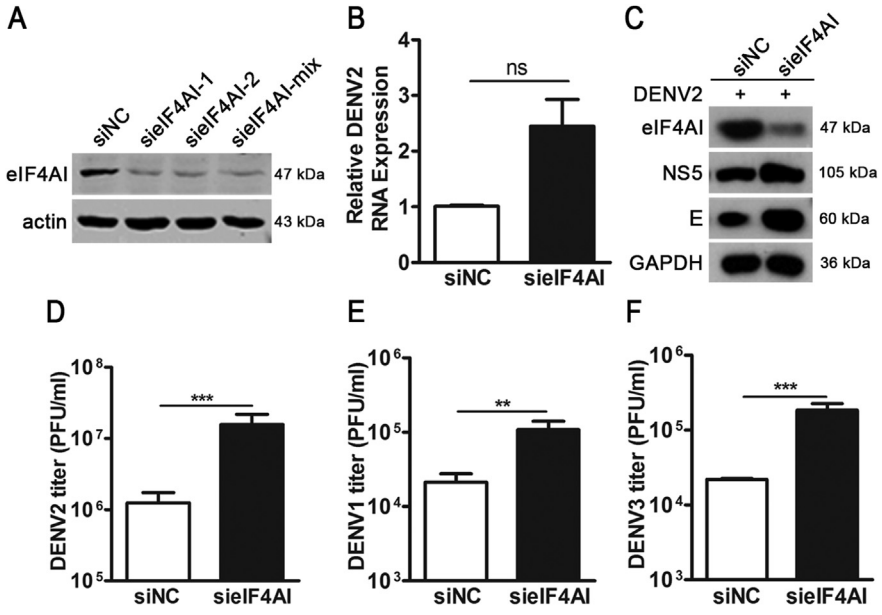

Fig. 3. EIF4AI silencing upregulated the replication levels of DENVs. A549 cells were transfected with siNC or sieIF4AI and harvested for Western blot at 48 h post transfection. Actin was loaded as an internal control (A). siRNAs transfected cells were mock-infected or infected with DENV2 (MOI 2). Cells were harvested for real-time PCR to measure viral RNA level (B) or Western blot to detect the protein levels of DENV2 NS5 and E (C). GAPDH was loaded as an internal control. Cell supernatants were harvested for TCID50 assay (D). (E,F), A549 cells were transfected with siNC or sieIF4AI, followed by DENV1 (E) or DENV3 (F) infection (MOI 2). Cells supernatants were harvested for TCID50 assay. Data were representative of three independent experiments.

ATP binding domain and helicase domain were involved in its association with NS4A.

3.3. EIF4AI negatively regulated the replication of Dengue virus

To examine whether eIF4AI plays a role in Dengue virus replication, we employed RNAi to knockdown the eIF4AI protein and compared the DENV replication levels. The knockdown efficiency of two different siRNAs against eIF4AI (sieIF4AI-1 and sieIF4AI-2) or their mixture (sieIF4AI-mix) were examined by Western blot. A significant reduction of eIF4AI expression level was observed in cells transfected with sieIF4AI-1, sieIF4AI-2 or sieIF4AI-mix than in those transfected with control siRNA (siNC)-transfected cells (Fig. 3A), indicating that the knockdown of these siRNAs was effective. In the following assays, we chose to use sieIF4AI-mix for eIF4AI knockdown.

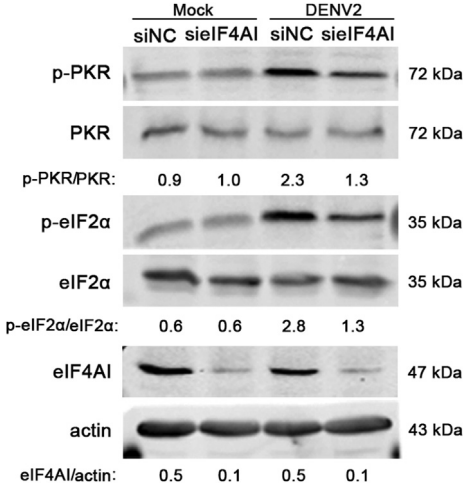

Fig. 4. EIF4AI was involved in the phosphorylation of PKR and eIF2α. A549 cells were transfected with siNC or sieIF4AI, followed by mock or DENV2 infection. Cells were harvested at 24 h p.i. for Western blot to detect the phosphorylated form and total protein levels of PKR and eIF2α. Actin was loaded as an internal control. The ratios of p-PKR/PKR, p-eIF2α/eIF2α, and eIF4AI/actin were calculated by Quantity One as shown below the representative blot of three independent experiments.

Then we compared the viral replication levels, including RNA expression level, protein accumulation and yield of infectious particles in eIF4AI-sufficient and eIF4AI-deficient cells by Real-time PCR, Western blot and TCID50 assay respectively. The viral RNA level was slightly but not significantly enhanced in eIF4AI-deficient cells ($p > 0.05$, Fig. 3B). And the expression levels of viral proteins, including a structural protein E and a nonstructural protein NS5 were significantly increased in eIF4AI knockdown cells (Fig. 3C). And the viral yield in eIF4AI knockdown cells was 10-fold higher than that in control cells ($p < 0.001$, Fig. 3D).

Furthermore, we assessed the role of eIF4AI in the replication of another two DENV serotypes, DENV1 and DENV3. Similarly, eIF4AI knockdown significantly increased the viral yields of DENV1 (7.0-fold, $p < 0.01$, Fig. 3E) and DENV3 (8.2-fold, $p < 0.001$, Fig. 3F) respectively. These data indicated that eIF4AI plays an antiviral role in the replication of DENVs.

3.4. EIF4AI was involved in the activation of PKR

As eIF4AI knockdown significantly upregulated the viral protein level and viral yield, we hypothesized that it plays a role in viral protein synthesis. And because one major mechanism of host cells to inhibit viral protein synthesis is through PKR and its substrate eIF2α [13,20,21], we tested whether eIF4AI has an impact on PKR activation. As expected, the phosphorylation levels of PKR and eIF2α in siNC-transfected cells were significantly enhanced by DENV2 infection, while in eIF4AI knockdown cells, the phosphorylation levels of PKR and eIF2α were significantly downregulated (Fig. 4). As a control, the protein levels of total PKR and eIF2α were comparable in all tested cells. These data suggested that the antiviral role of eIF4AI might exert, at least partially, through mediating the PKR activation.

4. Discussion

NS4A has been indicated to play multiple roles in Dengue virus replication, including inducing autophagy [4], evading the innate immunity [22], and inducing the cell membrane modifications to harbor the viral replication complex [5]. To better illustrate the roles of NS4A, we have attempted to isolate cellular proteins associated with NS4A. Current work uncovered that eIF4AI is a NS4A-interacting partner and has an anti-DENV effect.

First, eIF4AI was identified to interact with NS4A by Mass spectral analysis, and their interaction was validated by co-IP assay using both ectopic and endogenous proteins. Moreover, both N- and C- terminal domains of eIF4AI were found to be associated with NS4A, which can provide a close association as the NS4A is a small protein with four internal hydrophobic regions in membrane. Our work together with previous findings demonstrated that NS4A interacts with a variety of cellular proteins (eIF4AI, vimentin and PTB) and viral protein (NS4B) [23], underscoring the significance of NS4A in viral replication.

Unexpectedly, cellular translational protein eIF4AI plays an antiviral, rather than a proviral role in the replication of DENVs, including DENV1, DENV2 and DENV3. Apparently, the requirements for eIF4AI vary in different viruses: translation of some picornavirus is dependent of eIF4AI [13,14,16,24–29]; while other viruses such as Bovine viral diarrhea virus (BVDV) and hepatitis C virus (HCV) could bypass the requirement for eIF4AI [27,28]. Our observation that Dengue virus does not require eIF4AI for its replication was in keeping with previous work showing Dengue virus utilizes a non-IRES-mediated mechanism for translation initiation when cellular translation factors were limited [30,31]. In addition, another two members of *flaviviride* family, BVDV and HCV, can translate by an internal ribosome entry mechanism [27,28]. These observations indicate that many viruses have developed their own strategies to translate viral proteins independent of eIF4AI [32,33].

Furthermore, our results revealed that eIF4AI was involved in the phosphorylation of PKR and eIF2α. Antiviral protein PKR stays in an inactivated state in latent cells and becomes activated upon viral infection through dimerization and autophosphorylation [34,35]. Although in *in vitro* kinase reaction, PKR can be activated by dsRNA alone, many cellular proteins including the protein activator of PKR (PACT) [36], NF90 [37] and IPS-1 [19] have been reported to facilitate its activation in a cell context. Here our work revealed that eIF4AI might be an additional factor involved in PKR activation, and the underlying mechanism will be an interesting topic to be further investigated. Taken these data together, we deduced that the antiviral role of eIF4AI against Dengue virus might be exerted through PKR and eIF2α [13,20,21]. However, the possibility that eIF4AI has a direct negative effect on viral RNA synthesis could not be completely ruled out as the RNA level of DENV2 was slightly upregulated.

In summary, we proposed that a new role of NS4A in DENV replication is to interact with antiviral eIF4AI. And because Dengue virus has the ability to synthesize protein independent of eIF4AI and the cap-binding complex [30], the major biological significance of NS4A to recruit eIF4AI might be blocking eIF4AI from activating PKR and eIF2α. Therefore, our study identifies a new cellular protein that interacts with NS4A, eIF4AI, and illustrates a potential mechanism how DENV antagonizes cellular antiviral protein.

Author contributions

Conceived and designed the experiments: PZ, XYC, JX, JFH. Performed the experiments: XYC, JX, QRZ, YW, LQF, JYL. Analyzed the data: XYC, JX, PZ, JFH. Wrote the paper: XYC, PZ. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no conflict of interest.

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